Specificity and Selectivity in Galacto-Oligosaccharide Synthesis Reactions Catalyzed by β-Galactosidases

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ABSTRACT

Galacto-oligosaccharides (GalOS) are known as functional foods or prebiotics, and they have potential functions as efficient growth factors for Bifidobacteria and Lactobacilli. The Bifidobacteria participate in human digestion by improving metabolism as well as preventing the growth of putrefactive bacteria. The Bifidobacteria also posses anticarcinogen and anticholesterolaemic properties. In order to give the availability of GalOS in human diet, it will be necessary to manufacture synthetic GalOS using a simple method. This research is an attempt to synthesize GalOS by means of high concentrations of galactose or lactose as the substrate employing b-galactosidases (EC 3. 2. 1. 23) from Aspergillus oryzae, and Bacillus circulans. The characteristics of synthesis reaction of GalOS with these enzymes and the structure of GalOS products will be also observed.

All experiment steps will be carried out in the laboratory consisting of (a) synthesis of homoGalOS using b-galactosidases at various concentrations (20%-50% w/v) of galactose or lactose and at various pH (6.0 – 7.0); (b) synthesis of heteroGalOS with galactose as a donor and various acceptors (glucose, mannose, maltose, and sucrose) in the ratio of 1 : 1 using b-galactosidases; (c) determination of the amount of GalOS produced; and (d) determination of the structure of GalOS.

The experimental results showed that all enzymes were potential catalysts for the synthesis of GalOS. Reaction time significantly affected the amount of GalOS produced, where the exponential production occurred at the first 6 hours of incubation, and then it gradually increased to reach equilibrium after 24 hours incubation. The production of GalOS increased with the concentration of galactose or lactose up to 50% and then decreased when the concentration of lactose or galactose was higher than 50%. The synthesis reaction was not significantly affected by changing the pH from 6.0 to 7.0. The structure of homoGalOS and heteroGalOS consisted of various b-galactosidic linkages (b-1,3-; b-1,4-, and b-1,6-) clearly explaining that A. oryzae b-galactosidase was a non linkage-specific enzyme. The GalOS with b(1-4)-galactosidic were predominantly produced using b(1-4)-galactosidase from Bacillus circulans thus explaining that this enzyme is more specific than the enzyme from A. oryzae .

Keywords: prebiotic, galactooligosaccharides, b-galactosidase, A. oryzae, B. circulans

INTRODUCTION

A number of oligosaccharides are currently produced commercially by means of microorganisms. The use of microbial enzymes involving on the synthesis of novel oligosaccharides has been reported to be more simple and rapid than the chemical methods (Warren, 1995; Murata and Usui, 1997). One of the potential enzymes for oligosaccharide synthesis is b-Galactosidase (EC 3.2.1.23) which has been isolated and purified from different origin of microorganisms (Murata *et al.*, 1997; Thiem and Farkas, 1999). This enzyme has an ability to synthesize galactooligosaccharides (GalOS) through either equilibrium-controlled synthesis (reverse hydrolysis) or kinetically-controlled synthesis (transglycosidation) reactions.

Basically, more than 130 GalOS have been identified in human breast-milk. The total amount of these complex oligosaccharides is between 3 - 6 g/l. The human breast-milk GalOS have important functions as the inhibitors of bacterial adhesion to epithelial surfaces, an initial stage of infective process. These oligosaccharides are also considered to be soluble receptor analogues of epithelial cell surfaces participating in the non-immunological defense system of breast milk-fed infants (Kunz and Rudloff, 1996). It is reasonable that the breast milk-fed infants have more immune and defense system than the non breast milk-fed infants. The non fed-infants can not have these important GalOS from their diet or cow milk due to the difference on composition and characteristics compared to the breast-milk. The addition of synthetic GalOS to their diet will possibly improve their immune and defense system.

It is a great of interest in producing the breast-milk oligosaccharides rather than extracting these oligosaccharides from the breast-milk itself. This milk is not commercially available and it is also difficult to collect the milk from breast-feeding mothers. These oligosaccharides can be synthesized either by chemical or enzymatic techniques. The enzymatic techniques are simple and only need b-galactosidase, galactose, lactose and water to commence the reactions.

The mechanisms of GalOS formation using lactose as a substrate in the presence of b-galactosidase has been reviewed (Zarate and Lopez-Leiva, 1990). The b-galactosidases from different original sources have been employed for GalOS such as from *Bacillus circulans* (Murata *et al.*, 1997; Thiem and Farkas, 1999) and *Thermus aquaticus* (Berger *et al.*, 1995a; 1995b). Enzyme galactosidases have been immobilized onto different materials (Sungur and Akbulut, 1994; Nakanishi *et al.*, 1983) and then employed for oligosaccharides synthesis.

n, Man-α(1→2)Man-α(1→2)Man, and Man-α(1→2)Man-α(1→2)Man-α(1→2)Man as anti-infective agents (Suwasono and Rastall, 1996). Moreover, manno-oligosaccharides have been synthesized using immobilized α-(1-2)-mannosidase although this enzyme exhibited lower regio-selectivity (Suwasono and Rastall, 1998).

According to the above theory, an excellent technique on *in vitro* production of GalOS must be investigated. In this experiment, linkage-specific and non linkage-specific β-galactosidases will be employed for homoGalOS production. The linkage-specific β-(1-4)galactosidases is highly specific for cleaving non-reducing terminal galactose residues of oligosaccharides in a β(1-4) linkage. While the non linkage-specific β-galactosidases cleaves β-(1-3), β-(1-4), and β-(1-6) linkages. The reason of using these two enzymes is to observe the specificity and selectivity of the enzymes in the formation of novel GalOS with various glycosidic linkages, so that the product spectra of GalOS can be extended.

MATERIAL AND METHODS

MATERIALS

Chemicals used in the experiment were b-lactose (Sigma L-3750), D-Galactose (E-Merck 4061), D-Glucose (Sigma G-8270), D-Mannose (Sigma M-4625), Maltose (Sigma M-5885), Sucrose (Sigma S-9378), Maltotriose (Fluka 63430), Trehalose (Sigma T-5251), b-galactosidase (E.C. 3.2.1.23) from *Aspergillus oryzae*

(Sigma G-5160), b-galactosidase from *Bacillus circulans* (Wako 077-04331), *p*-nitrophenyl-b-galactopyranoside (Sigma N-1252), *o*-nitrophenyl-b-galactopyranoside (Sigma N-1127), distillated water, buffer standard pH 4.0 and pH 7.0.

Small equipments used were eppendorf tubes, volumetric pipettes, micro pipettes, erlenmeyer, beaker glass, reaction tubes, analytical balance, autoclave, incubator, pH meter, and spectrophotometer.

METHODS

Activity of β-Galactosidase from *Aspergillus oryzae* and *Bacillus circulans*

β-galactosidase was reacted with 5 mM *o*-NP-β-galactopyranoside or *p*-NP-β-galactopyranoside as the substrate in 100 mM sodium acetate buffer pH 6.0 and then incubated at 37°C for 10 minutes. Enzymatic reaction was cut by the addition of 100 mM sodium carbonate, and the amount of released *o*-NP or *p*-NP as a yellow solution was determined by spectrophotometer at 420 nm against the blank. One unit of enzyme was defined as the amount of enzyme releasing one µmole *o*-NP or *p*-NP per minute at 37°C.

Synthesis of HomoGalOS using β-Galactosidases

Synthesis reactions of homoGalOS in eppendorf tubes were performed at different concentrations of total galactose and lactose as the substrate (20%-50% w/v) in 100 mM sodium acetate buffer solution at various pH (pH 6.0 and pH 7.0). To each reaction mixture, b-galactosidase (10 Units) was added and the reaction mixture was incubated at 50°C for 144 hours. After incubation, the sugar solution was heated at 100°C for 2 minutes to inactivate the enzyme and the GalOS products were analyzed by HPLC.

Synthesis of HeteroGalOS Using β-Galactosidases

The synthesis of heteroGalOS was performed at 50% (w/v) total sugar consisting of a mixture of galactose and an acceptor namely glucose, mannose, maltose, and sucrose) in the ratio of 1 : 1 (w/w), in 100 mM sodium acetate buffer pH 6.0. To each reaction mixture, β-galactosidase (10 Units) was applied, and the reaction mixture was incubated at 50°C for 96 hours. The heteroGalOS product yields were analyzed by HPLC.

Analysis of GalOS Products by High Pressure Liquid Chromatography

GalOS products were quantified by HPLC equipped with refractive index detector and then using an Aminex-HPX 87 H column (300 mm x 7.8 mm) from BioRad eluted by deionized water at 50°C and the flow rate of 1.5 ml /min.

Analysis of GalOS Structures by Gas Chormatography-Mass Spectrometer

Prior to analyse the structure, GalOS was purified by gel filtration on a column (2.5 x 150 cm) of BioGel P-2 (Low Pressure Liquid Chromatography) from BioRad eluted with degassed deionized water at 0.5 ml/min. Carbohydrates were detected by refractive index detector. All fractions containing carbohydrates were collected and lyophilized prior to structural analysis. Product structures were analyzed by methylation, hydrolysis, reduction, and acetylation steps to produce partially methylated alditol acetates (PMAA). The PMAA were analyzed on a HP-5 capillary column (50 m x 0.32 mm) at 100-250°C (3°C per minute temperature programme) with flame ionisation detection. Mass spectra were recorded with a mass selective detector calibrated from 33 to 400 amu with a scan rate of 1.9 scans per second. The electron impact ionization current was set up at 50 mA.

RESULTS AND DISCUSSION

HOMOGALACTO-OLIGOSACCHARIDES (HOMOGALOS)

The synthesis reactions of homoGalOS were performed using galactose and lactose as the substrate at various concentrations (20% - 50% w/v) in buffer solutions at various pH (6.0 and 7.0), and these reaction mixtures were incubated in the incubator at 50°C for few days. β-Galactosidase from *Aspergillus oryzae* and

Bacillus circulans with the activity of 10 Unit per ml were used as the catalyst in the synthesis reaction.

From the experimental results, it is clear that the exponential production of homoGalOS occured during the first six hours reaction, and then gradually increased until twelve hours reaction before achieving equilibrium after twenty four hours incubation. The synthesis reaction of disaccharides ($GalOS_2$) using galactose as the substrate in the presence of *A. oryzae* β-galactosidase either at pH 6.0 or pH 7.0 showed similar patterns (**Figure 1** and **2**). Furthermore, $GalOS_2$ yields were higher when lactose was applied in the reaction as the substrate (**Figure 3** and **4**). In general, the profile of synthesis reactions employing *B. circulans* β-galactosidase was similar to that using *A. oryzae* β-galactosidase (Figures are not shown).

When lactose was used the substrate, the enzyme was very active not only in hydrolysis lactose to be glucose and galactose, but also in synthesis disaccharides ($GalOS_2$) and trisaccharides ($GalOS_3$). There were possible reactions occurring during synthesis reaction using lactose as presented in **Figure 5**. In the presence of small water activity, lactose was initially hydrolyzed to be galactose (Gal) and glucose (Glu) (1), and Gal

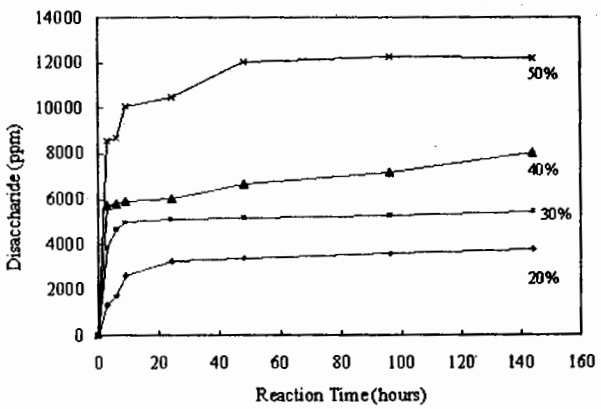

Figure 1. $GalOS_2$ Synthesis Using **Galactose** at **pH 6.0** Catalyzed by *Aspergillus oryzae* β-Galactosidase

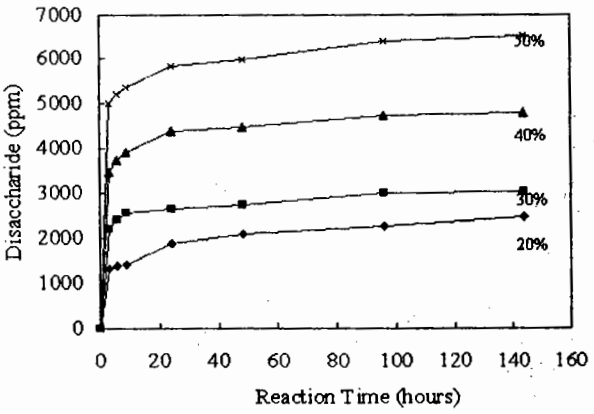

Figure 2. $GalOS_2$ Synthesis Using **Galactose** at **pH 7.0** Catalyzed by *Aspergillus oryzae* β-Galactosidase

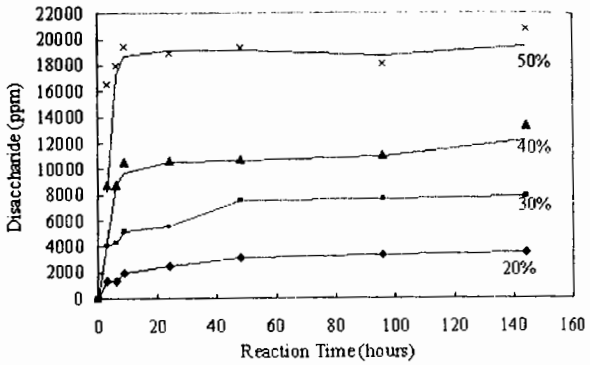

Figure 3. $GalOS_2$ Synthesis Using **Lactose** at **pH 6.0** Catalyzed by *Aspergillus oryzae* β-Galactosidase

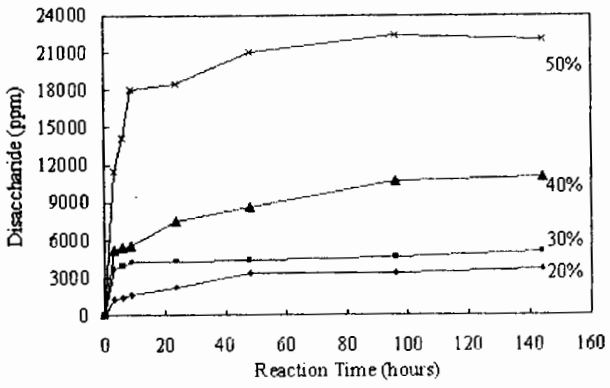

Figure 4. $GalOS_2$ Synthesis Using **Lactose** at **pH 7.0** Catalyzed by *Aspergillus oryzae* β-Galactosidase

Lactose (Gal-Glu) + H_2O	→	Gal + Glu	(1)
Gal-Glu + Gal	→	Gal-Gal + Glu	(2)
Gal-Glu + Gal	→	Gal-Gal-Glu	(3)
Gal + Glu	→	Gal-Glu	(4)
Gal + Gal	→	Gal-Gal	(5)
Gal + Gal-Gal	→	Gal-Gal-Gal	(6)
Gal-Glu + Glu	→	No synthesis	(7)

Figure 5. Schemes of Synthesis Reactions Using Lactose (Gal-Glu) as A Substrate Catalyzed by β-Galactosidase

then becomed a donor and Gal-Glu becomed an acceptor to form disaccharide (2) and trisaccharide (3). Glu resulted from hydrolysis (1) acted as an acceptor for Gal to produce disaccharide (4). Excessive Gal itself formed disaccharide (5), while synthesis would not occur when Glu acted as a donor with free hydroxyl side C-1 (6).

Gal + Gal	→	Gal-Gal	(8)
Gal + Gal-Gal	→	Gal-Gal-Gal	(9)
Gal-Gal + H_2O	→	Gal + Gal	(10)
Gal-Gal-Gal + H_2O	→	Gal + Gal-Gal	(11)

Figure 6. Schemes of Synthesis Reactions Using Galactose (Gal) as A Substrate Catalyzed by β-Galactosidase

The synthesis reaction using galactose as a substra occurred as presented in **Figure 6**. The use of hig concentration of galactose had driven the reaction t form disaccharide (8) and trisaccharide (9). Howeve small hydrolysis changed disaccharide to be Gal (1(and trisaccharide to be Gal and Gal-Gal (11).

The pH of reactions was set closely to a neutral p in order that synthesis reaction in the future could b performed in normal water. The difference of pH di not affect the formation of homoGalOS, especially whe lactose was used as the substrate employing *A. oryza* β-galactosidase (**Table 1**). Using galactos disaccharides synthesized by *A oryzae* enzyme at pl 7.0 were lower than those produced at pH 6.0. It i probably due to partial hydrolysis of disaccharides (1(to form trisaccharides (9). Using *B. circulans* enzym disaccharide yields produced at pH 6.0 was very hig so that these repressed the formation of trisaccharide (**Table 2**).

From the above results, it seems that both enzyme have similar ability in the synthesis of GalOS althoug the reactions were performed at different pH. This con dition suggested that the enzymes involving in the syn thesis reaction were naturally stabilized with the pres ence of a high concentration of galactose or lactose pro viding the protection on each active side of the enzym from the environment caused by different ionizatio states.

Table 1. The Quantity of Galactooligosaccharides Produced by ***Aspergillus oryzae*** β-Galactosidase after 48 hours incubation

Product (ppm)	Substrate Galactose (w/v)				Substrate Lactose (w/v)			
	20%	30%	40%	50%	20%	30%	40%	50%
Disaccharide ($GalOS_2$)								
pH 6	3390	5145	6661	12062	3064	7590	10739	19376
pH 7	2086	2743	4468	5986	3362	4382	8603	19693
Trisaccharide ($GalOS_3$)								
pH 6	6735	7017	7233	7343	6817	7795	8700	11309
pH 7	6901	7136	7496	8789	6386	7111	8965	11555

Table 2. The Quantity of Galactooligosaccharides Produced by ***Bacillus circulans*** β-Galactosidase after 48 hours incubation

Product (ppm)	Substrate Galactose (w/v)				Substrate Lactose (w/v)			
	20%	30%	40%	50%	20%	30%	40%	50%
Disaccharide ($GalOS_2$)								
pH 6	3306	3475	3428	4226	11429	15080	25731	31078
pH 7	3038	3382	3455	3523	12198	15183	19040	28618
Trisaccharide ($GalOS_3$)								
pH 6	441	893	1064	1416	2547	3098	4337	7321
pH 7	484	778	991	1403	4203	5309	9746	18055

The yield of GalOS increased with increasing concentration of galactose or lactose. Water activity at 20% substrate was still high and it would motivate hydrolysis reaction to occur so that there was a strong competition between hydrolysis and synthesis reactions. Higher concentration of sugars until a certain level were required to reduce water activity in order to drive equilibrium-synthesis reactions. However, at more than 50% total sugar, the water activity was too low for the enzyme to be active in producing GalOS since the enzyme might be dehydrated. When the reaction was set up with 20% to 50% substrate, disaccharides and trisaccharides could be obtained as the products. Unfortunately, no tetrasaccharides could be detected from the reaction.

STRUCTURE OF HOMOGALOS

The product mixture of GalOS obtained from the synthesis reaction was separated into mono-, di-, and trisaccharide fraction by means of gel chromatography on Bio Gel P2. The fractions of disaccharide ($GalOS_2$) and trisaccharide ($GalOS_3$) were collected and then concentrated by freeze-drying. The structure of GalOS was determined by methylation, hydrolysis, reduction, and acetylation to be partially methylated alditol acetates (PMAA). Derivatives of $GalOS_2$ and $GalOS_3$ were quantified and identified by applying the PMAA to gas chromatography-mass spectrometry. The mass spectra were compared to the standard (Jansson *et al.*, 1976; Carpita and Shea, 1989). From $GalOS_2$ fraction, the spectra 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylgalacitol represents the nonreducing terminal galactose, and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylgalacitol represents 1,4-galactosidic linkage. These two spectra of $GalOS_2$ product indicated that the product should be Gal β(1→4)Gal.

It is clear from the experiment that β-galactosidases originated from *Aspergillus oryzae* and *Bacillus circulans* catalysed the synthesis of $GalOS_2$ and $GalOS_3$ with several linkages (**Table 3**). When galactose was used as a substrate in the synthesis reaction employing *A. oryzae* β-galactosidase, $GalOS_2$ product contained a mixture of β(1→3)-, β(1→4)-, β(1→6)-galactosidic linkages. While in the reaction using B. *circulans* enzyme, $GalOS_2$ consisted of β(1→4)- and β(1→6)-galactosidic linkages. These patterns were not similar to those presented by the reaction using lactose as the substrate in the presence of *A. oryzae* or B. *circulans* β-galactosidase from which β(1→4)-galactosidic linkage was found exclusively.

Furthermore, it seems that the enzyme from *Bacillus circulans* is more linkage-specific than that from *Aspergillus oryzae*. *Bacillus circulans* β-galactosidase showed its specificity and selectivity in the synthesis

Table 3. Structural Estimation of HomoGalOS Products Synthesized by *Aspergillus oryzae* and *Bacillus circulans* β-Galactosidases.

Enzyme source	Substrates	Disaccharides	Trisaccharides
Aspergillus oryzae	Galactose	Gal β(1→3)Gal Gal β(1→4)Gal Gal β(1→6)Gal	Gal β(1→4)Gal β(1→3)Gal Gal β(1→4)Gal β(1→6)Gal
	Lactose	Gal β(1→4)Gal Gal β(1→4)Glu	Gal β(1→4)Gal β(1→3)Gal Gal β(1→4)Gal β(1→4)Glu
Bacillus circulans	Galactose	Gal β(1→4)Gal Gal β(1→6)Gal	Gal β(1→4)Gal β(1→4)Gal
	Lactose	Gal β(1→4)Gal Gal β(1→4)Glu	Gal β(1→4)Gal β(1→4)Gal Gal β(1→4)Gal β(1→4)Glu

reaction using lactose as the substrate to produce $GalOS_2$ and $GalOS_3$ having an exclusive β(1→4)-galactosidic linkage. The specificity of this enzyme is similar to that of *Thermus aquaticus* β-galactosidase (Berger *et al.*, 1995a; 1995b).

HETEROGALACTOOLIGOSACCHARIDES (HETEROGALOS)

Heterogalactooligosaccharides (heteroGalOS) were synthesized using galactose as a donor sugar and other several sugars as acceptors (glucose, mannose, maltose, and sucrose) (**Table 4**). The total sugar of each reaction mixture was 50% (w/v) and it was then incubated for 96 hours by an assumption that this condition would have reached equilibrium. The product from a reaction between galactose and glucose is not always called lactose since the product may consist of various galactosidic linkages (β(1→3)-, β(1→4)-, and β(1→6)-). When galactose was reacted with other monosaccharides (glucose and mannose), the expected main product yielded was disaccharides (**Figure 7** and **8**). As mentioned by Zarate and Leiva (1989) that the formation of oligosaccharides occurred via transgalactosylation with hydroxyl acceptor of sugar. If the acceptor was monosaccharide, the product would be disaccharide (reaction 1) on **Figure 7** and **Figure 8**). However, it can be explained from these two figures that disaccharide might be formed due to the excessive amount of galactose (reaction 2), and trisaccharide might be formed due to the excessive amount of galactose (reaction 3) or binding between disaccharide and acceptors (reaction 4).

Furthermore, the reaction of galactose and disaccharides (maltose and sucrose) was expected to produce trisaccharides (**Figure 9** and **10**) where the yield of trisaccharides was smaller than that of disaccharides (**Table 4**). The formation of trisaccharides might be due to the binding between galactose and acceptors (reaction 1) or the excessive amount of galactose (reaction 3), while disaccharides would be from galactose or sucrose itself (reaction 2).

From methylation analysis of heteroGalOS (**Table 5**), *Bacillus circulans* β-galactosidase showed high regioselectivity in forming β(1→4)-galactosidic linkages when galactose was reacted with glucose and maltose. On the otherhand, the enzyme from *A. oryzae* had a tendency toward the formation of β(1→6)-galactosidic linkage which was like most glycosidases tended to cleave the primary hydroxyl group of acceptors and formed β(1→6)-linkage between donor

Tabel 4. Heterogalactooligosaccharides from Reaction Mixtures Containing Galactose and Various Acceptors in The Presence of *A. Oryzae* and *B. Circulans* β-Galactosidases.

Enzyme Source	Acceptors	Products	Disaccharides (ppm)	Trisaccharides (ppm)
Aspergillus oryzae	Glucose	Galactosylglucose	14.702	7.117
	Mannose	Galactosylmannose	16.735	6.775
	Maltose	Galactosylmaltose	40.964	12.013
	Sucrose	Galactosylsucrose	18.903	9.399
Bacillus circulans	Glucose	Galactosylglucose	6.774	6.407
	Mannose	Galactosylmannose	6.939	6.385
	Maltose	Galactosylmaltose	76.372	10.887
	Sucrose	Galactosylsucrose	9.977	6.138

Table 5. Structural Estimation of HeteroGalOS Products Synthesized by *Aspergillus oryzae* and *Bacillus circulans* β-Galactosidases Using Galactose as The Substrate.

Enzyme source	Donor	Deduced Linkage	Area Relative (%)	Product Ratio(%)
Aspergillus oryzae	Glucose	t-gal 6-glu	75 25	Gal β(1→6)Glu = 100
	Mannose	t-gal 4-man 6-man	18.05 41.19 40.76	Gal β(1→4)Man = 50.26 Gal β(1→6)Man = 49.73
	Maltose	t-gal 4-glu 6-glu	41.87 41.51 16.62	Gal β(1→6)Gluα (1→4)Glu = 100
	Sucrose	t-gal 6-glu 2-gal	29.50 33.45 37.05	Gal β(1→6)Gluα (1→2)Fru = 100
Bacillus circulans	Glucose	t-gal 4-glu	52.67 47.33	Gal β(1→4)Glu = 100
	Mannose	t-gal 4-man 6-man	28.58 37.79 33.63	Gal β(1→4)Man = 52.90 Galβ(1→6)Man = 47.10
	Maltose	t-gal 4-glu	25.83 74.17	Gal β(1→4)Gluα (1→4)Glu = 100
	Sucrose	t-gal 6-glu 2-gal	31.99 17.51 50.50	Gal β(1→6)Gluα (1→2)Fru = 100

and acceptor (Ichikawa *et al.*, 1992). It is also clear from **Table 5** that the formation of GalOS products are obviously dependent on the enzyme regioselectivity. A similar study on regioselectivity performed using α-mannosidase showed that α-mannosidase from Jack bean had lower regioselectivity compared to that from *Aspergillus phoenicis* (Suwasono and Rastall, 1998). The regioselectivity of enzyme in synthesis reactions is clearly dependent on the enzyme origins, the structure and the anomeric configuration of acceptors (Bucke *et al.*, 2000).

CONCLUSION

Microbial β-galactosidases originated from *Aspergillus oryzae* and *Bacillus circulans* have potency as the catalyst for producing galactooligosaccharides using high concentrations of galactose and lactose as the substrate. The higher yield of galactooligosaccharides were produced by the higher concentration of substrate used until a certain level where the enzyme was dehydrated and loss its potency. High concentration of substrates stabilized the enzyme and protected the active side from ionization changes. Exponential production of galactooligosaccharides occurred during the first six hours reaction, and then equilibrium condition was achieved after twenty our hours reaction. Enzyme β-galactosidase from *Bacillus circulans* showed higher specificity and selectivity, compared to the enzyme from *Aspergillus oryzae*, in forming β(1→4)-galactosidic linkage particularly when galactosyl donor bonding with glucosyl acceptor. It can be suggested that β-galactosidase from *Bacillus circulans* is a linkage-specific enzyme and β-galactosidase from *Aspergillus oryzae* is a non linkage-specific enzyme.

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